A Laboratory Manual of
Chemical Pathology

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PREFACE

THIS manual is an account of the methods used in the Department of Chemical Pathology, St Thomas's Hospital Medical School; they have been carefully selected from the literature on the basis of criteria which are discussed in Chapter 1.

Alternative methods are given for a few substances but we have not attempted to compile an exhaustive review of all the techniques available: indeed, it is probable that the huge numbers of new methods which are reported annually make a comprehensive review too cumbersome for ordinary use. For similar reasons we have not included any interpretations of the results.

The methods selected have proved to be satisfactory for the routine work of the Department and most of them appear to be suitable for use in the field of chemical pathology in routine clinical practice.

We are indebted to Dr R. V. Brooks for the section on steroid determinations.

CONTENTS

GENERAL SECTIONS

1

CHOICE OF METHODS

THE ideal chemical determination would have good reproducibility, specificity, sensitivity, accuracy, and would require only simple apparatus and moderate technical skill. Unfortunately, these criteria are rarely combined. The methods given in this manual are selected from the large number available after extensive trial and consideration of the above desirable features.

(a) **Reproducibility**

Perhaps the most important practical requirement of any method is that it should give consistent results, even though there may be some loss during determination or inclusion of other substances in the final result. Acquaintance with the method and its limitations, and with the changes in the results which are due to disease, can lead to interpretations of these results which are reliable aids to diagnosis and assessment of progress.

(b) **Specificity**

The inclusion of an unknown and perhaps variable proportion of non-specific substances in the results, however, is not only inelegant but is potentially misleading. With increasing refinement of clinical interpretation, the trend is rightly towards greater specificity.

Few substances have properties which are specific enough to permit their direct determination in mixtures as complex as biological fluids. In fact, among the methods included in this manual, only flame photometry for sodium and potassium can be said to be direct determinations, since there is no need to separate the constituents of the original fluid. For nearly all the other substances some isolation process is required.

The commonest is the removal of proteins from the original solution; this is necessary because proteins have so many properties that they are capable of reacting with most of the reagents employed for the determination of other substances, i.e. they "interfere" with the reaction and render it non-specific. In other methods, e.g. the determination of calcium, an attempt is made to isolate the substance itself by precipitating it as an insoluble compound which may be purified by centrifuging and washing. Such processes, although necessary with present methods, are undesirable because they

introduce the risk of three sorts of error: the isolation process may not completely remove the undesirable substance, it may remove some of the desired substance, and it leads to volumetric errors from the pipetting required.

It should be noted that, even after the removal of obvious interfering substances, some methods are still non-specific. Such methods remain in use either because better techniques are not yet available or because specific techniques are too complex and time-consuming for routine use.

(c) Sensitivity

On the sensitivity of a method depends the accuracy of the final measurement. If the final measurement is made by titration, the titration figure should be at least 0·80 ml. from a microburette or 4·0 ml. from a larger burette, in order to reduce volumetric errors to less than 2%. If the final measurement is made by colorimetry, the optical density should lie within the optimum range for the instrument used; for standard colorimeters this range is from 0·2 to 0·6.

It will be appreciated that sensitive methods allow the adjustment of the final titration or colorimetry to fit the above criteria, since, if the measurement should be too high at first, it may be reduced by using less of the original material. With an insensitive method, the final figure will be too low for accuracy and cannot usually be raised by using more of the original material because this will also introduce more of the interfering substances or, for instance, prevent adequate precipitation of protein.

In a few cases, insensitive methods have to be accepted because none better are yet available (e.g. plasma creatinine and creatine) but their use has been avoided wherever possible.

(d) Overall accuracy

An accurate method must be reproducible, specific and sensitive, and these points have been taken into account with all the methods; it has been noted above that no method is ideal and that some compromise is inevitable.

The assessment of the accuracy of a method is made in three ways: (1) The method is used for the determination of a substance in solutions of known concentration; the results obtained by the method should repeatedly be within 1% of the correct answers but, with specially difficult substances, one may be forced to accept wider limits of error. If a method fails with pure solutions, it is not worth proceeding with; even if it gives correct results at this stage, it may fail with biological mixtures and is therefore taken through the second stage of testing.

(2) Known quantities of a substance are added to biological material and carried through the method. The percentage recovery of the substance is then calculated, and should be within 3% of the correct result.

It should be noted that accurate recoveries by the method are essential, but even if they are obtained there may be a constant error in all the results.

(3) Comparison with results obtained by other methods is often the final consideration in assessment of accuracy. If the results are close to those given by an accepted method, they will be taken as accurate. On the other hand, if they differ from those given by accepted methods, prolonged experience may be needed to decide which is correct. The emphasis, in this manual, is on methods of proved accuracy.

(e) Other considerations

Speed, the availability of equipment, and the degree of technical skill required have to be considered. In general, speed has not been an important consideration with us because it usually conflicts with accuracy and this is considered to be the basic essential. The methods chosen, however, are not unreasonably slow, neither do they demand unusual degrees of skill from technicians. For some determinations an ultra-violet spectrophotometer has been employed because it appears to offer special advantages of accuracy or specificity or because it is the only means of determination; where possible, alternative methods are described.

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PREPARATION AND USE OF STANDARD SOLUTIONS

THREE types of standard solution are used in the laboratory.

(*a*) Volumetric standard solutions used in acid-alkali titrations, e.g. the use of oxalic acid to standardize sodium hydroxide. These are described in greater detail in the following section.

(*b*) Volumetric standard solutions used in oxidation–reduction titrations, e.g. the use of potassium iodate to standardize sodium thiosulphate (see p. 211) and the use of oxalic acid to standardize potassium permanganate (see p. 92).

(*c*) Specific standards such as phosphorus, cholesterol, sodium and potassium, with which unknown solutions are compared every time the method is used; details of their composition and preparation are given in the individual methods. In some methods it is not practical to include a standard with every determination. Alternatives are either to construct a standard curve to which reference is made for each determination, or to use an artificial standard.

PREPARATION OF STANDARD SOLUTION

Principles

1. A primary standard solution of any of the three types above must be prepared from an accurately weighed amount of pure substance. This implies that the substance must be a solid able to be dried in a desiccator and not take up moisture during the weighing. It may need to be recrystallized. The accurately weighed substance is dissolved and transferred quantitatively to a volumetric flask. When the transfer is complete and the solution is at room temperature its volume is brought up to the mark on the flask with distilled water or other reagent if this is required.

2. Secondary volumetric standard solutions are made from substances which do not fulfill the conditions required for accurate weighing but may be standardized by titration against a primary standard.

3. Standard solutions should not be stored in volumetric flasks but at once transferred to clean dry bottles. It is a wise precaution to rinse out the bottle a few times with a little of the standard solution before transferring the whole. The bottles should be clearly labelled and dated. Every care should be taken

to ensure that no standard solution is allowed to evaporate or become contaminated, hence the following rules must be observed:

(*a*) Never leave a standard solution unstoppered.
(*b*) Never remove portions of the standard solution by putting pipettes into the bottle; instead, pour the approximate amount required into a clean dry beaker and pipette from this.
(*c*) Never return to the bottle portions of standard solution left over in beakers.

Technique

1. *Primary standard solutions*

(*a*) *Accurate weighing* — Set aside in beakers in a desiccator approximately 65 g of oxalic acid and 20 g of potassium iodate and weigh daily until the weight is constant; this requires three to seven days. For each substance weigh a 250 ml. beaker accurately to within 0·001 g, then weigh into it the required quantity of the desiccator-dried substance.

For 1 litre of 1·00 *N* oxalic acid weigh 63·025 g of $(COOH)_2 \cdot 2H_2O$.
For 500 ml. of 1·00 *N* potassium iodate weigh 17·836 g of KIO_3.

(*b*) *Quantitative transfer* — Add approximately 200 ml. of distilled water to the beaker and stir the contents with a glass rod, heating if necessary in order to dissolve all the solid. Pour the solution down the glass rod into the appropriate volumetric flask. Repeatedly add more distilled water to the beaker, wash its walls and pour into the flask. Wash down the glass rod with more distilled water and make up to the mark. If heating was required, time must be allowed for the solution to cool to room temperature before making up to volume. All quantitative transfers are made in this way.

2. *Secondary standard solutions*

(*a*) *Sodium hydroxide* —

(i) Prepare 2 litres of approximately 1 *N* sodium hydroxide by dissolving 84 g of NaOH in water and making up to 2 litres. Standardize this approximately 1 *N* solution with 1·00 *N* oxalic acid by pipetting 25 ml. of the acid into a conical flask, adding a few drops of de Wesselow's indicator, and titrating with the sodium hydroxide from a burette until the indicator changes from blue through slate grey to green. Repeat until two titrations agree within 0·05 ml.

$$\text{Normality of sodium hydroxide} = \frac{\text{normality of oxalic acid} \times \text{volume of oxalic acid}}{\text{volume of sodium hydroxide } (V).}$$

i.e. Normality of sodium hydroxide $= \frac{25}{V}$ and should be greater than 1·0.

(ii) Adjust the strength of this sodium hydroxide solution to 1·00 *N* by adding a calculated volume of distilled water to a known volume of the sodium hydroxide. Check the normality by titration as before.

(iii) Prepare 0·10 *N* sodium hydroxide by diluting 25 ml. of the 1·00 *N* solution to 250 ml. in a volumetric flask, using Grade A glassware.

(iv) Prepare 0·01 *N* sodium hydroxide by diluting 10 ml. of the 1·00 *N* solution to 1 litre in a volumetric flask, using Grade A glassware. This solution is unstable and requires checking at least once a week.

Note: Store all standard sodium hydroxide solutions in plastic bottles with plastic stoppers

(*b*) *Sulphuric acid* —

(i) Prepare 2 litres of approximately 1 *N* sulphuric acid by cautiously adding 60 ml. of concentrated sulphuric acid (sp. gr. 1·84) from a measuring cylinder to about 1500 ml. of distilled water. When cool make up to 2 litres with distilled water. Standardize this approximately 1 *N* sulphuric acid with 1·00 *N* sodium hydroxide by pipetting 25 ml. of acid into a conical flask with a few drops of de Wesselow's indicator and titrating with standard alkali from a burette until the indicator changes from blue to green. Repeat until two titrations agree within 0·05 ml. and calculate the normality as before.

(ii) Adjust the strength of this sulphuric acid to 1·00 *N* as described above and check by titration.

(iii) Prepare 0·10 *N* sulphuric acid by diluting 25 ml. of the 1·00 *N* solution to 250 ml. in a volumetric flask, using Grade A glassware.

(iv) Prepare 0·01 *N* sulphuric acid by diluting 10 ml. of the 1·00 *N* solution to 1 litre in a volumetric flask, using Grade A glassware.

USE OF STANDARD SOLUTIONS

1. *In methods where there is a direct stoichiometric relationship between the amount of substance and the final measurement*

In this type of method the final measurement is usually a titration of the unknown with a standard solution, e.g. titration of calcium oxalate with standard potassium permanganate. Here the concentration in the unknown is calculated from its chemical equivalent weight.

e.g. 1·0 ml. 0·01 *N* $KMnO_4 \equiv$ 1·0 ml. 0·01 *N* $Ca^{++} \equiv$ 0·2 mg Ca.

In such methods a standard is not taken through the same procedure as the unknown and hence errors due to inaccuracies in measurements and ageing of reagents are not under control. It is here particularly that there is need for a system of quality control.

2. *In methods involving comparison between the unknown and a standard solution to obtain the concentration in the unknown*

(*a*) In most colorimetric methods the final depth of colour in the unknown depends on a number of factors such as efficacy of reagents, time of colour development, temperature and pH besides the actual concentration of the substance. In other words there is no direct stoichiometric relationship between the concentration of the substance and the final measurement and it is necessary to include one or more standard solutions in each batch of analyses. It is assumed that variables which affect the colour developed by the unknown solutions will equally affect the standards. The concentration in the unknown can then be deduced from the standards.

(*b*) Similarly in flame photometric measurements an arbitrary setting of the instrument is made on a standard solution and the concentration in the unknown deduced by comparison.

(*c*) The possible sources of error are:

(i) calculation errors;

(ii) inaccuracies in technique;

(iii) ageing of reagents.

Errors in calculation are reduced to a minimum by arranging the concentration of standards and reagents so that the least possible number of steps in calculation are required.

Inaccuracies in technique and ageing of reagents are controlled to some extent by the fact that standards are taken through most of the procedure in the same way as the unknowns and it is soon apparent when the optical density of a standard shifts from the usual value. Quality control is an additional check on accuracy.

THE QUALITY CONTROL SYSTEM

The system of quality control as described by King and Wootton is used in this laboratory. Solutions of known composition are prepared by dissolving weighed amounts of pure, dry substance and are issued to technicians who are not aware of the concentration of the substance to be measured. The observed results are recorded on a chart as a percentage above or below the correct value. This procedure has the advantage of detecting any trend above or below the limits of error allowed for any particular determination. For most determinations aqueous solutions are satisfactory but for calcium and protein serum is needed. In this laboratory we use the standard freeze-dried serum prepared by Glaxo Laboratories Ltd. This freeze-dried serum has already been analysed for sodium, potassium, chloride, urea, phosphorus, uric acid, calcium and protein and it is a useful check on the methods in use in the laboratory to compare the results with those supplied with the serum.

Quality control solutions

1. SODIUM — *Stock solution* of sodium chloride 500 mEq/l.
   NaCl 14·6 g
   Distilled water to 500 ml.
   Preserve with a few drops of chloroform and store at room temperature.
   Control solution to contain 120 to 160 mEq/l.

2. POTASSIUM — *Stock solution* of potassium chloride 10 mEq/l.
   KCl 0·373 g
   Distilled water to 500 ml.
   Preserve with a few drops of chloroform and store at room temperature.
   Control solution to contain 2 to 7 mEq/l.

3. CHLORIDE — *Stock solution* of sodium chloride 500 mEq/l.
   Control solution to contain 70 to 120 mEq/l.

4. BICARBONATE — *Stock solution* equivalent to sodium bicarbonate 200 mEq/l.
   Na_2CO_3 2·12 g
   Distilled water to 100 ml.
   This solution is used because bicarbonate solutions are unstable. Store in the refrigerator.
   Control solution to contain 15 to 40 mEq/l.

5. UREA — *Stock solution* of urea 500 mg%
   Urea 2·50 g
   Distilled water to 500 ml.
   Store in the refrigerator.
   Control solution to contain 10 to 400 mg%.

6. PHOSPHORUS — *Stock solution* of potassium phosphate 100 mg% phosphorus
   KH_2PO_4 2·19 g
   Distilled water to 500 ml.
   Preserve with a few drops of chloroform and store at room temperature.
   Control solution to contain 2 to 6·5 mg% phosphorus.

7. GLUCOSE — *Stock solution* of glucose 1% in saturated benzoic acid
   Glucose 5·00 g
   Saturated benzoic acid to 500 ml.
   Store in the refrigerator.
   Control solution to contain 25 to 500 mg%.

8. CHOLESTEROL *Stock solution* of cholesterol 100 mg% in ethanol-acetone.
Cholesterol, 3 times recrystallized from ethanol 0·100 g
Ethanol-acetone (1 : 1) mixture to 100 ml.
Store in the refrigerator.
Control solution to contain 1 to 10 mg% to be treated as a 1 in 50 dilution of serum (i.e. equivalent to 50 to 500 mg% serum cholesterol).

9. CALCIUM Freeze-dried serum prepared by Glaxo Laboratories Ltd., Greenford, Middlesex
Distilled water 10 ml.
The calcium content in mg% of reconstituted serum is given with each batch of freeze-dried serum.

10. PROTEIN Reconstituted freeze-dried serum prepared as described above.

Limits of error

Sodium	±1·5%	Phosphorus	±5%
Potassium	±4%	Glucose	±5%
Chloride	±2%	Cholesterol	±5%
Bicarbonate	±3%	Calcium	±2%
Urea	±5%	Protein nitrogen	±3%

REFERENCE

KING, E. J. & WOOTTON, I. D. P.; *Micro-analysis in Medical Biochemistry*. Churchill, London (1956).